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13. ABSTRACT (Maximum 200 Words)
Patients with recessive dystrophic epidermolysis bullosa (RDEB) frequently present with squamous cell carcinomas (SCCs) probably as a result of chronic blistering and extensive scarring. These tumors are clinically aggressive as they metastasize readily. The metastasis-associated protein (MTA)-1, a transcription suppressor, is overexpressed in several epithelial neoplasms including SCCs. Our preliminary results demonstrate that MTA1 expression is induced by activation of the epidermal growth factor receptor (EGFR). As deregulation of EGFR signaling is frequently observed in aggressive epithelial neoplasms we propose to study the role of EGFR signaling and MTA1 expression in SCCs derived in RDEB patients. Our Specific Aims are to establish cell lines derived from SCCs in non-RDEB and RDEB patients, characterize the malignant phenotype of these cells as it relates to EGFR expression and signaling and to expression of MTA1, examine the contribution of EGFR/MTA1 to proliferation, invasiveness, and cell survival and identify EGFR-dependent signaling pathways contributing to MTA1 expression in these cells. The results from this research will provide invaluable tools for future analysis of the pathobiology of carcinoma cells and will ascertain whether EGFR/MTA1 signaling pathways contributes significantly to the metastasis and invasiveness of SCC derived from RDEB patients.

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INTRODUCTION

Epidermolysis bullosa (EB) is a group of heritable genetic skin blistering diseases caused by the disruption of the normal function of the basement membrane zone, a distinct structure that separates the upper epidermis from the underlying dermis (1). Skin blisters between the dermal/epidermal junction in EB are extremely similar to those caused by chemical warfare agents including sulfur mustard (SM, dichlorodiethyl sulfide). In the recessive dystrophic forms of EB (RDEB), mutations in collagen type VII gene disrupts the anchoring fibrils connecting the basement membrane to the underlying dermis, thus causing blistering below the lamina densa. The functional consequences of these mutations, which render the basement membrane zone defective, directly contribute to their clinical manifestations (2-4). As a result of chronic blistering, re-epithelialization, and extensive scarring, RDEB patients frequently develop highly invasive squamous cell carcinomas (SCC) (5-7). SCC is the second most common form of skin cancer generally associated with chronic ultraviolet light exposure, but they can also arise in association with persistent skin wounds as in the cases of RDEB (8,9). Despite the high frequency of these neoplasias in RDEB patients, very little is known about the molecular characteristics of these tumors in comparison to SCCs occurring spontaneously in individuals without EB.

The metastasis tumor antigen 1 (MTA1) is overexpressed in and correlates well with many highly metastatic cancers including human epithelial-derived breast and esophageal carcinomas (10-13). MTA1 is a member of the nucleosome-remodeling histone-deacetylase (NuRD) complex and interacts directly with histone deacetylase proteins 1 and 2 (HDAC1/2) (14-17). We previously demonstrated that MTA1 is overexpressed in SCCs relative to normal keratinocytes (18). Furthermore, we found that MTA1 expression is controlled by the activation of the epidermal growth factor receptor (EGFR) in this cell type. Forced overexpression of MTA1 in keratinocytes increases cell proliferation and invasiveness, characteristics evident in SCCs from RDEB.

Thus the overall goal of our research through DAMD17-02-1-0215 is to begin an in depth investigation of RDEB-derived SCCs and ascertain whether the epidermal growth factor receptor- (EGFR) and/or MTA1 mediated signaling pathways contribute significantly to the highly aggressive malignant phenotype these cells. In order to study the molecular characteristics and mechanisms of metastasis in these cancer cells, we proposed to establish cell lines derived from SCC of RDEB patients and to characterize the malignant phenotype (growth, invasiveness, survival potential, and dependence on EGFR/MTA1 activation) of these cells. Our **Specific Aim 1** is to establish and characterize epithelial keratinocyte SCC cell lines from RDEB and non-RDEB skin biopsies. **Specific Aim 2** is to characterize the malignant phenotype (growth, invasiveness, and survival potential) of these cells as it relates to EGFR expression and signaling and to expression of MTA1. **Specific Aim 3** is to examine the contribution of EGFR/MTA1 to proliferation, invasiveness, and cell survival. Finally, **Specific Aim 4** is to identify the signaling pathways downstream of EGFR activation that are relevant to MTA1 expression in SCC from RDEB and non-RDEB. Since malignant cancer is characterized by its metastatic and invasive properties, identifying and characterizing the genes that are involved in metastasis will provide the means to predict, select, and treat patients for aggressive therapy.

BODY

This progress report will focus on accomplishments covered in Specific Aims 1, 2, and 3. A portion of this work has been accepted to the Journal of Biological Chemistry (see Appendix). In addition, another manuscript is in preparation.

Establish and characterize keratinocyte cell lines from SCC biopsies obtained from RDEB and non-RDEB patients.

In order to study the biology of tumor cells from RDEB, we propose to establish these cells in culture. This is a critical part of this proposal because these cells will serve as excellent tools for any future studies involving keratinocyte tumor biology. Within this past funding year, we received approval from the Office of Regulatory Compliance and Quality for Human Subjects Protection (Department of the Army) and have begun to collect skin tumor samples from RDEB patients. Although RDEB patients are rare, we have successfully collected several tumors from these patients and are currently maintaining these cells in culture. We would require at least 3-5 distinct tumor cell lines.

We began studying the molecular characteristics that would distinguish RDEB-SCCs from spontaneous SCCs at the tissue level. We first examined the state of differentiation of these tumors by immunostaining for involucrin, a protein highly expressed in epithelial cells undergoing differentiation and those cells which lose their proliferative potential (19,20)(**Fig. 1A, a**). We examined the expression pattern and level of involucrin expression in RDEB-SCCs (**Fig. 1A, c and d**) and compared them to those of non-RDEB-SCCs (**Fig. 1A, b and c**). In normal human skin, involucrin was expressed in the upper differentiated cell layers of the epidermis. The basal cells are undifferentiated proliferative cells and thus did not express involucrin. However in both RDEB- and non-RDEB-SCCs, involucrin expression extended throughout all cell layers of the epidermis (**Fig. 1A, b-d**). Interestingly, the expression level of involucrin in RDEB-SCC was significantly lower than that of non-RDEB-SCCs. The results could suggest that RDEB-SCCs might be less differentiated than other SCCs. We are currently examining other differentiation markers such as transglutaminase, loricrin, keratin 1, keratin 10, and filaggrin. Antibodies against these proteins are commercially available.

To assess MTA1 protein expression, we obtained a commercially available polyclonal antibody specific for MTA1. By immunohistochemical analysis we observed an upregulation of MTA1 expression in RDEB-SCCs (**Fig. 1B, a**) compared to non-RDEB-SCCs (**Fig. 1B, b**). The staining of MTA1 appeared to be localized to the cytoplasm as well as nucleus. These preliminary results are very promising and provide evidence that SCCs derived from RDEB express a higher level of MTA1, suggesting a more metastatic phenotype. As mentioned below, we recently cloned a novel member of the MTA gene family, which we named MTA3. We raised antibodies against this protein and are currently examining the expression level of MTA3 in RDEB-SCCs.

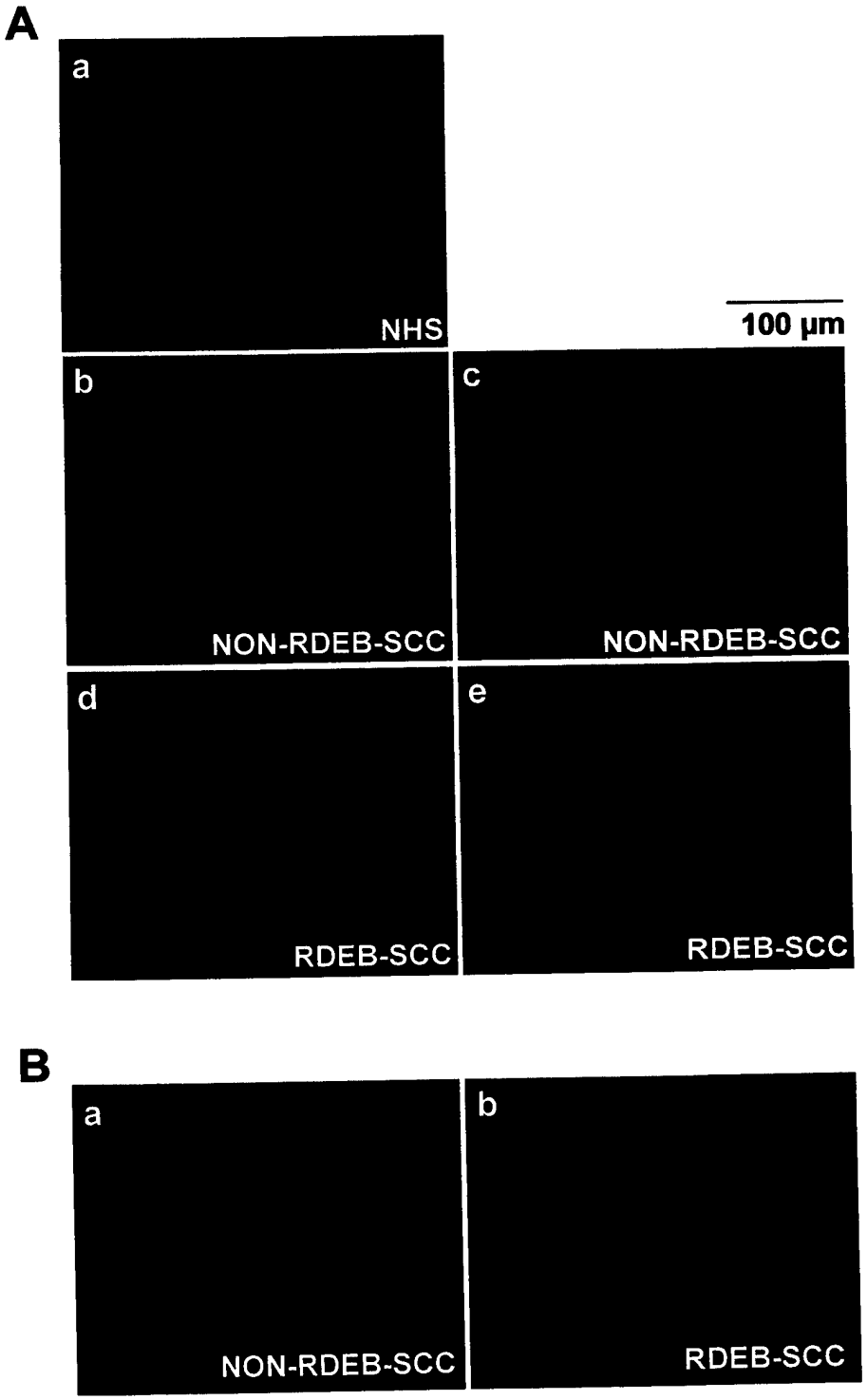

Figure 1. Molecular characterization of RDEB-SCC and non-RDEB-SCC. (A) A representative of each normal human skin, non-RDEB-SCC and RDEB-SCC are shown stained for the differentiation marker involucrin (green). In normal tissue, involucrin was expressed in the highly differentiated upper cell layers of the epidermis. Yellow dashed lines demarcate dermal/epidermal barrier. In both non-RDEBs and RDEB-SCCs, involucrin was detected in all cell layers. Interestingly, the expression level of involucrin appeared to be stronger in the non-RDEB-SCC. (B) Immunostaining for MTA1 (red) revealed more MTA1-positive cells in the RDEB-SCC compared to the non-RDEB-SCC. All tissues were stained for DNA with DAPI (blue).

Determine the EGF receptor-mediated signal transduction pathways regulating the expression of MTA1.

There is significant evidence supporting a relationship between aberrant EGF receptor activation and migratory/invasive properties of epithelial tumor cells. Interestingly epithelial-derived breast and esophageal carcinomas aberrantly express high levels of both EGF receptor and MTA1. It is our hypothesis that activation of the EGF receptor and its signal transduction pathways may regulate the expression of MTA1. We recently demonstrated that the EGF receptor contributes to steady-state expression of MTA1 mRNA and protein.

Similar to the role of EGF receptor in skin cancer, deregulation of the estrogen receptor stimulates mammary epithelial cell growth in breast cancer. We recently cloned a novel gene sharing high homology to both human and mouse MTA1. We named it MTA3 and raised antibodies specific against this protein. MTA3 is highly expressed and localized to both the cytoplasmic and nuclear compartments of breast and skin tumor cells. In collaboration with Dr. Rakesh Kumar (Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX), we demonstrated that MTA3 plays a role in regulating the estrogen receptor-mediated epithelial-to-mesenchymal transition (manuscript in press; see attachment). The tumor progression of mammary epithelial cells is associated with the down regulation of cell adhesion components such as E-cadherin, mediated through the zinc-finger transcriptional repressor Snail. Similar to MTA1, MTA3 expression is mediated through the estrogen receptor in breast epithelial cells (21). MTA3 directly represses Snail and thus regulates E-cadherin expression. Both the estrogen receptor and MTA1 can bind to regulatory elements within the MTA3 promoter, thus resulting in dynamic complex modulation of the estrogen receptor regulation of MTA3 expression and the epithelial-to-mesenchymal transition.

One of the goals of this proposal is to study the role of MTA1 in modulating cell proliferative potential. It has been documented that hypoacetylation of histones favors transcriptional silencing, a potential underlying cause of cell proliferation and cancer development. MTA1 has been shown to interact directly with and enhance the histone deacetylase activity of HDAC1/2 in the NuRD complex. We previously demonstrated that MTA1 regulates growth, invasiveness, and survival potential of normal epidermal keratinocytes. Here we report a novel role for MTA1 in regulating cellular processes, that when go awry, would favor carcinogenesis. By yeast two-hybrid protein-protein interaction system and in vitro or in vivo binding assays, we observed the direct interaction between MTA1 and REV7 (UV revertible gene) (**Fig. 2A&C**) and MAD2 (mitotic arrest-deficient) (**Fig. 2C**) but not the negative control protein lamin C (**Fig. 2D**). REV7 interacts with the spindle assembly checkpoint protein MAD2 (mitotic arrest-deficient), which regulates cell cycle progression into anaphase (22,23). In non-mitotic cells, MTA1 was found localized to the nuclei (**Fig. 2E, top panels**). However, during division as cells undergo mitosis, MAD2 has been shown to bind to the kinetochores of chromosomes and MTA1 was detected co-localized to microtubule spindles (**Fig. 2E, bottom panels**). Epithelial carcinomas overexpressed MTA1 as well as MAD2. Epithelial cells lacking MTA1 demonstrate reduction in cell cycle progression and upregulation of MAD2. These results demonstrate the diverse roles of MTA1 from nucleosome remodeling by enhancing histone deacetylation to DNA repair and spindle assembly checkpoint during mitosis.

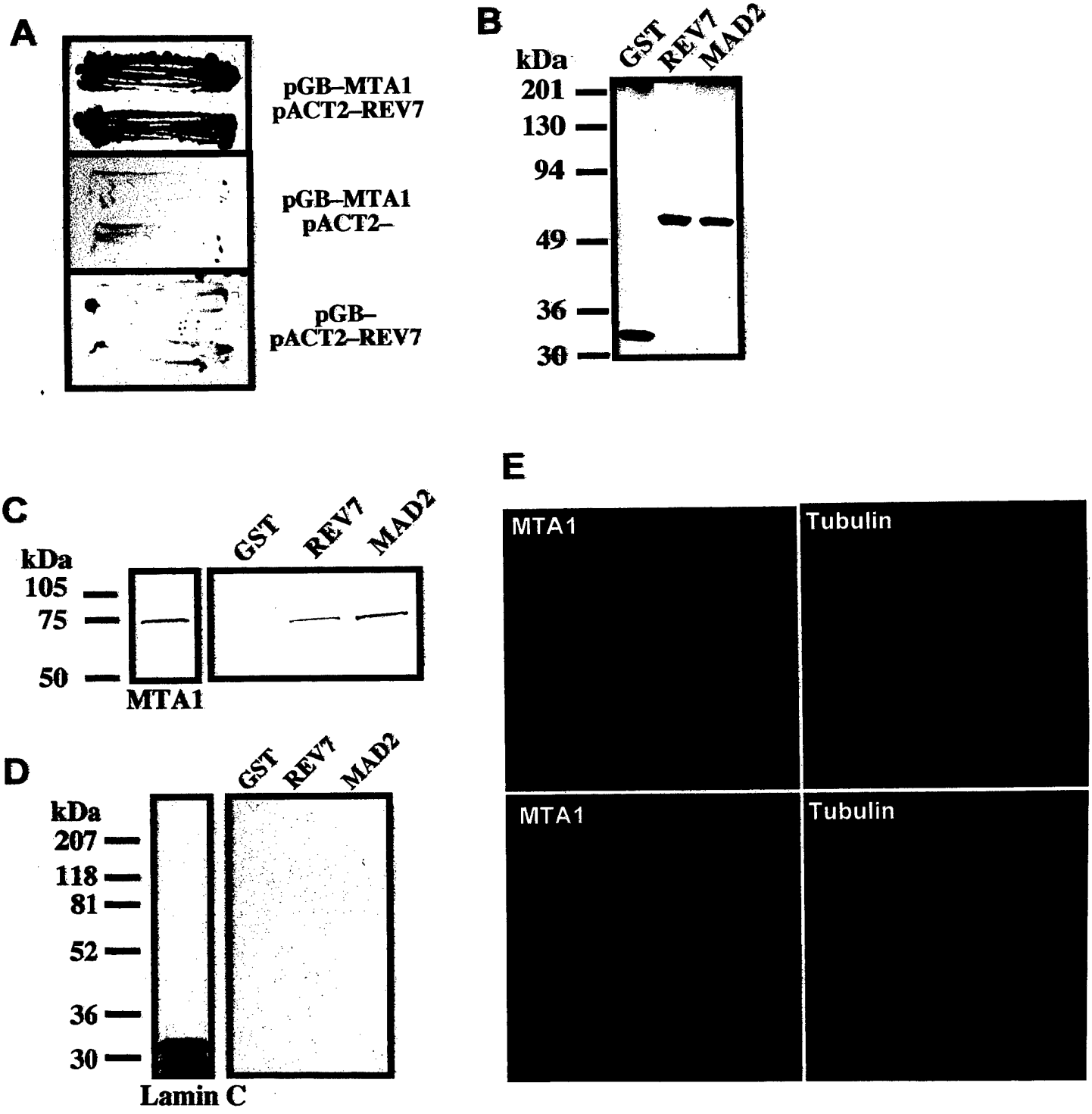

Figure 2. Molecular interaction of MTA1 with REV7 and MAD2 and potential role of MTA1 during mitosis. (A) Yeast two hybrid system revealed interaction between the MTA1 bait and the REV7 protein. (B) We generated bacterially produced recombinant glutathione S transferase fusion proteins of REV7 and MAD2. Western blotting with anti-GST antibodies. (C) GST pull down assay to demonstrate that MTA1 interacted with REV7-GST and MAD2-GST but not GST alone. (D) GST, REV7-GST, and MAD2-GST did not interact with a non-specific control protein, Lamin C. (E) Immunofluorescent staining of MTA1 (red) and α-tubulin (green) in non-mitotic (top panels) and mitotic (bottom panels) cells.

KEY RESEARCH ACCOMPLISHMENTS

• Establishment of several RDEB-SCC epithelial cell lines derived from highly advanced skin carcinomas. Initial molecular characterization demonstrated that these tumors were less differentiated as compared to non-RDEB-SCCs. However, RDEB-SCCs expressed significant high level of MTA1, a molecular marker for invasive potential.

• Cloning of novel member of the MTA1 gene family, MTA3. Collaboration with Dr. Kumar has resulted in a published work demonstrating that MTA3 expression is regulated through the estrogen receptor. The estrogen receptor and MTA1 can bind to regulatory elements within the MTA3 promoter resulting in a dynamic complex cyclical modulation of the estrogen receptor regulation of MTA3 expression and the epithelial-to-mesenchymal transition. Potential role of MTA3 in EGF receptor mediated signaling pathway needs further examination.

• Novel role of MTA1 in DNA repair and mitotic spindle assembly checkpoint, cellular processes that when go awry, would favor carcinogenesis. The results provided are preliminary and during years 3-4 of this funded research, we will further assess mechanism regulating this interaction and the role of EGF receptor signaling pathway in regulating this interaction.

REPORTABLE OUTCOMES

Some of the results mentioned above have been included in a publication (see attached appendix).

CONCLUSIONS

During the second year of this funding we have successfully collected RDEB tumor tissues and established several tumor cell lines derived from these samples (Specific Aims 1 and 2). This will be the main focus of year 3 funding. Our discovery of novel member of the MTA gene family, MTA3, was not in the original design of the proposal, but it has profoundly enhanced our understanding of the complex roles of MTA gene family in cancer progression. We will characterize the complex role of both MTA1 and MTA3 estrogen receptor mediated epithelial/mesynchymal transition.

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APPENDIX

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Upstream Determinants of Estrogen Receptor-α Regulation of MTA3 Pathway*

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#These authors contributed equally to this study

Running title- Regulation of MTA3 gene expression by estrogen receptor

Keywords: MTA3, Coregulators, Transcription, Chromatin

[1]The abbreviations used are: MTA, metastatic tumor antigen; ER, estrogen receptor-alpha; luc, luciferase; mAb, monoclonal antibody.

ABSTRACT

Although recent studies have shown a role of estrogen receptor (ER) in the regulation of epithelial-to-mesenchymal transition (EMT) via MTA3, the role of upstream determinants of ER regulation of MTA3 and the underlying molecular mechanism remains unknown. Here we show that *MTA3* gene regulation by ER is influenced by dynamic changes in levels of nuclear coregulators. *MTA3* promoter has a functional ER element half-site with which MTA1 and HDACs interact under basal conditions. Upon estrogen stimulation, these corepressors are derecruited with concomitant recruitment of ER, leading to increased MTA3 transcription and expression. Genetic inactivation of MTA1 promotes the ability of ER to upregulate MTA3 expression, while knockdown of ER enhances MTA1 association with *MTA3* gene. Modulation of ER functions, by corepressors (i.e., MTA1 and MTA1s) or coactivators (i.e., AIB1 and PELP1/MNAR), alters ER recruitment to *MTA3* chromatin, MTA3 transcription, and expression of downstream EMT components. These studies provide novel insights into the transregulation of the *MTA3* gene and reveal novel roles of upstream determinants in modifying the outcome of MTA3 axis and cell differentiation.

INTRODUCTION

The development of human breast cancer is promoted by estrogen stimulation of mammary epithelial cell growth. ER alpha (ER) is the major estrogen receptor in the human mammary epithelium. Binding of estrogen to ER triggers conformational changes that allow ER to bind to the 13-base-pair palindromic estrogen response element (ERE) in the target gene promoters and stimulates gene transcription, thereby promoting the growth of breast cancer cells. Transcriptional activity of ER is affected by a number of regulatory cofactors including chromatin-remodeling complexes, coactivators, and corepressors (1, 2,3).

Recent findings have demonstrated that the NuRD-70 polypeptide of the nucleosome remodeling complex is identical to metastatic tumor antigen 1 (MTA1) (4,5) and that MTA1 physically interacts with HDAC1/2 (6,7). The *MTA1* gene is shown to correlate well with the metastatic potential of several human cell lines and cancers, including breast cancers (8-11). Using *in vitro* models, Mazumdar et al. have shown that MTA1 interacts with ER and represses ER-transcription by recruiting HDAC to the ERE-containing target gene chromatin in breast cancer cells (12) and that MTA1- overexpressing breast cancer cells exhibit aggressive phenotypes (13). MTA1s, another family member, is a naturally occurring variant of MTA1 that contains a novel sequence of 33 amino acids with one potential nuclear receptor binding motif, LRILL. MTA1s inhibits ER nuclear signaling by sequestering ER in the cytoplasm but enhances ER cytoplasmic signaling and thus promotes tumorigenesis (14).

One of the principal phenotypic changes in breast cancer metastasis is the increased tendency of the cancer cells to undergo epithelial-to-mesenchymal (EMT) transition that is characterized by reduced expression and consequently, functions of cell-adhesion components such as E-cadherin (15-17). The zinc-finger transcriptional repressor, Snail, mediates the

repression of E-cadherin expression and leads to the inhibition of Snail function in epithelial cells, thus, restoring the expression of E-cadherin as well as cell-to-cell junctions (18,19). Recently, Fujita et al. identified MTA3 as an ER-regulated gene and showed that MTA3 upregulation prevents EMT by directly repressing Snail, and thereby, upregulating E-cadherin (20). Although these observations highlight the significance of EMT in breast cancer invasiveness and suggest a complex role for MTA family members in modifying ER functions in breast cancer cells, the precise mechanism by which ER regulates MTA3 expression and the putative nature of upstream determinants of MTA3 expression remain poorly understood.

Here we show that the MTA3 gene contains an ER element half-site, and that both ER and MTA1 are recruited to the same site in the MTA3 promoter chromatin. Further, estrogen stimulates MTA3 promoter activity in a corepressor-sensitive manner. In addition, modulation of ER functions by corepressors (i.e., MTA1 and MTA1s) or coactivators (i.e., AIB1 and PELP1/NMAR) results in the suppression or stimulation of ER recruitment to the MTA3 chromatin; and consequently, affects the expression of EMT components. Together, these studies reveal that dynamic changes in the levels of ER coregulators influence ER regulation of MTA3 and reveal a novel role for nuclear coregulators in modifying the outcome of MTA3-mediated EMT.

EXPERIMENTAL PROCEDURES

Cell lines and Reagents. MCF-7 human breast cancer cells and Hela cells were maintained in Dulbecco's modified Eagle's medium-F12 (1:1) supplemented with 10% fetal calf serum HeLa cervical cancer cells were obtained from the American Type Culture Collection (Manassas, VA). MCF7 clones stably expressing MTA1, MTA1s and PELP1 have been

described earlier (12,14,21). Steroid hormone E2, tamoxifen and charcoal-stripped serum (DCC serum) was purchased from Sigma Chemical Co. ICI-182, 780 was purchased from Tocris, Ellisville, MO. Antibodies against MTA1 were purchased from Santa Cruz Inc. (SantaCruz, Calif) and T7 mAb was procured from Novagen (Milwaukee, Wis). Antibodies for E-Cadherin were obtained from Zymed and ER antibody was purchased from UBI. Snail antibody used here has been described before (22).

Anti-peptide rabbit against MTA3 was generated against the mouse MTA3 amino acids 420 – 438 (sequence: SDEEKSPSPTAEDPRARSH). The MTA3-GST fusion protein was run on SDS-PAGE and transferred to nitrocellulose filter. The blot was stained with ponceau and the region of the blot with the GST-MTA3 fusion protein was cut. The nitrocellulose pieces were incubated in 10 mL of 1% BSA for 3-4 hr at 4°C to block nonspecific sites. The BSA treated nitrocellulose pieces were incubated with diluted rabbit antiserum in cold overnight. Subsequently, the nitrocellulose pieces were washed 4 x in TBST for 5 min each, and transferred to eppendorph tubes and the bound MTA3 antibody was eluted by sequential incubatoin with 300 μL of MAPS buffer pH 3.0 for 5 min. Immediately after elution 200 μL of 1M TRIS pH 9.5 was added to prevent denaturation of the antibody. The eluates were pooled and stored -20°C.

Cloning of MTA3 Promoter and MTA3 cDNA : To clone the MTA3 promoter, we first identified the BAC clone (ID#RP11-314A20) containing the MTA3 genomic region using human genome sequence information. We then purchased this BAC clone from BACPAC Resources (Children's Hospital Oakland Research Institute, CA). Two fragments (1078 bp and 735 bp) of MTA3 promoter region were amplified by polymerase chain reaction (PCR). The amplified products were cloned into the PGL3 luciferase reporter vector (Promega Corp.

Madison, WI) using *SacI* and *Xho1* sites. The sequence of the construct was verified by comparing its sequence with that in the human genome database. PCR based cloning was used to generate the deletion construct and point mutation of the 1078 bp MTA3 promoter. PCR product of MTA3 cDNA was cloned at pcDNA3.1A using *KpnI* and *XhoI* sites (see Table I for primer sequences).

Reporter Assays: For the reporter gene transient transfections, cells were cultured for 24 h in minimal essential medium without phenol red containing 5% DCC serum. The MTA3-luciferase (luc) reporter constructs were transfected using FuGENE 6 according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Twenty-four hours later, the cells were treated with E2 for 16 h. The cells were then lysed with a passive-lysis buffer, and the luc assay was performed using a luc reporter assay kit (Promega Corp. Madison, WI). The total amount of DNA used in the transfections was kept constant by adding a parental vector. Each transfection was carried out in six-well plates in triplicate wells.

Chromatin Immunoprecipitation Assay (ChIP): Approximately 10^6 cells were treated with 1% formaldehyde (final concentration, v/v) for 10 min at 37^0C to cross-link histones to DNA. The cells were washed twice with phosphate buffered saline, pH 7.4 containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). ChIP assay was performed as described previously (12). Either an ERα–specific antibody, MTA1 mAb or T7 mAb were used for immunoprecipitation of protein-bound chromatin and precipitated DNA was amplified by PCR using primers flanking the proximal half ERE site (see Table I for primer sequences). The amplified fragment was sequence verified.

Gene knockdown by siRNA. ERα specific siRNA and control non-specific siRNA were purchased from Dharmacon. For MTA1 knockdown 4-for-Silencing siRNA Duplexes were designed using Qiagen program and synthesized at Qiagen. SiRNA trasfections were carried out using 20 μM of pooled siRNA duplexes and by using 4 μl of Oligofectamine (Invitrogen) according to maufacturer's protocol in six well plates. After 72 hours, cells were prepared for ChIP assay or western blotting.

Immunofluorescence and confocal studies: The cellular location of proteins was determined using indirect immunofluorescence, as described previously (14). In brief, MCF7 cells were plated on glass coverslips in 6-well culture plates. When the cells were 50% confluent, cells were rinsed with phosphate-buffered saline, fixed in cold methanol for 6 min, then processed for immunofluorescence staining of endogenous MTA3, E-Cadherin or Snail. Cells were counterstained with ToPro3 to visualize the nucleus. Slides were further processed for imaging and confocal analysis using a Zeiss LSM 510 microscope and a x 40 objective.

RESULTS AND DISCUSSION

Cloning and Regulation of MTA3 Promoter by Estrogen: To delineate the mechanism of ER regulation of MTA3 expression, we first analyzed the sequence of the putative MTA3 promoter region (Genebank genomic sequence accesion # NT_022184) for the presence of ER responsive elements using Matinspector (Genomatix). This program did not reveal any

consensus 13 bp ERE sites. However, we found that the MTA3 promoter contains three potential ERE half-sites (TGACC) (Fig. 1A). Interestingly, all the three ERE half-sites were localized in the vicinity of AP1 binding sites. Since a number of ER responsive genes have been shown to be regulated via ERE half-sites in conjunction with either AP1 or SP1 sites (21), we examined the potential involvement of these ERE half-sites in the regulation of MTA3 expression by ER.

To explore the recruitment of ER to the endogenous MTA3 promoter, MCF-7 breast cancer cells were treated with or without estrogen and subjected to chromatin immunoprecipitation (ChIP) assays using an anti-ER antibody. We found that ligand-activated ER is recruited to the endogenous MTA3 promoter at the ER element half-site from positions -1256 to –1245 but not to the other two potential sites (Fig. 1B). To verify these results, we next designed a pair of primers that encompass proximal as well as the middle potential ERE half-sites. Results showed estrogen-induced recruitment of ER to the MTA3 promoter region of expected 714 bp (Fig. 1B, right panel). To ascertain that the detected 239 bp band indeed represents the regulatory region corresponding to the proximal ERE half-site, the PCR amplified DNA fragment was cloned into a TOPO vector and confirmed by sequencing (data not shown). Estrogen-induced recruitment of ER to the MTA3 promoter chromatin was effectively blocked by the inclusion of anti-estrogen ICI-182780 suggesting that estrogen-activated recruitment of ER to the MTA3 gene was specific. Since ER recruitment to the MTA3 promoter was also inhibited by the protein-synthesis inhibitor cycloheximide (Fig. 1C), it appears that this event requires new protein synthesis and the noted effects could be mediated via an indirect mechanism. This appears to be consistent with the notion that ER action through an ERE half-site requires an associated factor and that direct interaction with DNA may not be involved (21). Given that it has been previously demonstrated that estrogen upregulates both its own receptor,

ER as well as ER coactivators expression (21, 24), it seemed likely that sufficient quantities of ER and its coactivators were necessary for upregulation of MTA3 expression by estrogen. To test this hypothesis, we looked at the levels of ER under the same experimental conditions and did not find changes in ER amounts in the cell for any of the conditions.

To further study the regulation of the MTA3 promoter via the ERE half-site, a MTA3 promoter fragment was amplified from the BAC clone and DNA fragments of desired sizes, 1078 bp and 735 bp, were obtained. The MTA3 promoter fragments were cloned into pGL3-luciferase (luc) reporter system. The functionality of MTA3-luc vectors was tested in MCF-7 and HeLa cells (Fig. 2A, 2B). Estrogen treatment of cells stimulated the MTA3-regulatory element-driven reporter activity from the 1078-bp fragment (-1528 to -450; contains two ERE half sites, at –1459 and at -1241) but not from the 735 bp fragment (-1185 to -450; lacks -1241 bp ERE half-site) (Fig. 2B), and therefore, a MTA3-1078 bp luciferase construct was used in the subsequent studies. Estrogen-stimulated MTA3 promoter activity was effectively blocked by tamoxifen, an estrogen antagonist, which also had a modest inhibitory effect on basal MTA3 promoter activity (Fig. 2C, 2D). To validate that the proximal ERE half site (at –1241) in estrogen stimulation of MTA3 promoter, we created a point mutation until –1241 and deletion at –1450 of the 1078 bp MTA3 promoter. As shown in Fig. 2E and 2F, the point mutation at the proximal ERE half site abolished the estrogen stimulation, where as deletion of the middle ERE half-site did not affect estrogen stimulation as compared to the 1078 bp fragment. Together, these findings confirm that the observed recruitment of ligand-activated ER to the MTA3 promoter chromatin is accompanied by increased MTA3 promoter activity.

Regulation of MTA3 Expression and EMT by MTA1: Since MTA1 and MTA1s are natural inhibitors of ER functions (12,14,25), we next tested whether deregulation of these

proteins may influence MTA3 expression and functions. Using breast cancer cells stably expressing T7-MTA1 (12) or T7-MTA1s (14), we found that repression of ER function by MTA1 or MTA1s abolished the ability of estrogen to promote the recruitment of ER to the *MTA3* gene chromatin (Fig. 3A and 3B). Since both MTA1 and MTA1s had similar effects, we used only MTA1 to repress the functions of ER in subsequent studies. Overexpression of MTA1 in breast cancer cells also resulted in the inability of estrogen to induce MTA3 mRNA (Fig. 3C). The observed inhibition of MTA3 expression by MTA1 was at the level of transcription, as co-expression of MTA1 inhibited both the basal and estrogen-induced stimulation of MTA3 promoter activity (Fig. 3D). In MTA1 overexpressing conditions without estrogen stimulation, expression of MTA3 protein was not inhibited while MTA3 promoter activity did show repression. Based on these results, one could speculate that there could be additional regulatory elements which may be involved in MTA3 basal expression in the physiological setting. It could also be possible that the basal repression observed in the promoter-reporter analysis is due to an inherent limitation of the assay system since it reflects the regulation of the promoter area largely in isolation and under artificial conditions. Results from confocal scanning microscopy also demonstrated that MTA1 deregulation leads to a significant reduction in the levels of nuclear MTA3, upregulation of Snail and consequent downregulation of E-cadherin as compared to the levels of MTA3, Snail and E-cadherin in control vector transfected cells (Fig.4). All of the above findings suggest that inhibition of ER transactivation functions by MTA1 could impair the ability of ER to upregulate MTA3 expression.

Modulation of MTA3 chromatin by ER coregulators: Since MTA1 prevented the recruitment of ligand-activated ER to the MTA3 promoter, we next investigated the possibility of MTA1 recruitment to the MTA3 promoter chromatin. MCF-7 cells were treated with or

without estrogen, and chromatin lysates were immunoprecipated with anti-MTA1 antibody. We found that MTA1 associates with the MTA3 promoter in unstimulated MCF-7 cells but not in estrogen-stimulated cells (Fig. 5A). MTA1 association with the MTA3 promoter was found to be at the proximal ERE-half site, which was also immunoprecipated in the ER CHIP assay. These observations suggest that MTA1 associates with the basal MTA3 promoter chromatin and upon estrogen treatment, is derecruited with simultaneous recruitment of ER to the ER element half-site in the MTA3 promoter chromatin. Since MTA1 has been shown to physically interact with HDAC1 and HDAC2 (6), we next examined whether the HDACs also interact with the basal MTA3 promoter. Results from ChIP studies using anti-HDAC antibodies show the association of the HDACs with the MTA3 promoter, which, like MTA1, were also derecruited from the MTA3 promoter upon estrogen treatment (Fig. 5B). These findings raise possibility that the basal MTA1 association with the MTA3 promoter might influence the status of EMT components, presumably due to the inhibitory effect of MTA1 on the ER transactivation functions. To test this notion, we silenced endogenous MTA1 expression using siRNA and examined the status of MTA3 target genes, as well as ER target genes in MCF-7 cells. Results indicated that inhibition of MTA1 expression leads to the downregulation of Snail and the upregulation of E-cadherin (Fig. 5C). Interestingly, there was also significant upregulation of progesterone receptors PR-A and PR-B, well-established ER target gene products, thus suggesting enhanced ER transactivation functions due to reduced levels of MTA1 and, consequently, loss of corepressor functions of MTA1. Consistent with these findings, reduction in the levels of MTA1 by siRNA was accompanied both by increased basal expression of MTA3 and enhanced ability of estrogen to upregulate MTA3 expression (Fig. 5D). Interestingly, there was also increased estrogen-induced stimulation in the levels of ER in MTA1-knockdown cells (data not shown), implying

that in the absence of MTA1, ligand-induced upregulation of ER might participate in the amplification of ER regulation of MTA3 expression.

Regulation of MTA3 gene by the endogenous MTA1. To determine whether the above changes in the levels of MTA3 and its targets in MTA1-knockdown MCF-7 cells were due to modification of the MTA3 chromatin in the vicinity of the ER element half-site, we next performed ChIP analysis of ER recruitment on the MTA3 chromatin under conditions of MTA1 knockdown (Fig. 6A). We found that suppression of endogenous MTA1 expression leads to detectable basal association of ER with the MTA3 promoter, which, as expected, was further enhanced upon estrogen signaling. The above result was corroborated in a luciferase assay system where knockdown of MTA1 resulted in significant increase in both basal and estrogen-induced MTA3-luciferase activity (Fig. 6B). Consistent with these results, silencing of the endogenous MTA1 in MCF-7 cells resulted in a substantial decrease in the recruitment of HDACs to the MTA3 promoter segment and estrogen stimulation led to complete dissociation of the HDACs from the MTA3 promoter (Fig.6 C). To further support the notion that MTA1 and ER might be competing for the ER element half-site in the MTA3 promoter, we next knocked down the endogenous ER in MCF-7 cells by siRNA, as assessed by the levels of ER and the ER target gene products, PR-A and PR-B (Fig.6D). As before, cells were also treated with a control siRNA. Next we performed a ChIP assay to analyze the status of MTA1 recruitment on the MTA3 promoter under conditions of ER knockdown. We found that ER silencing indeed leads to a significantly increased association of endogenous MTA1 with the basal MTA3 chromatin. Results also suggest that estrogen stimulation was unable to trigger derecruitment of MTA1 from the MTA3 promoter region (Fig 6E). Together, these experiments establish that the MTA3

promoter is a target of MTA1 and that manipulation in the levels of MTA1 expression influences the recruitment pattern of ER to the MTA3 promoter chromatin in a significant manner.

In conformity with a recent report (20), we found that estrogen signaling increases MTA3 protein levels (Fig. 7B) in a dose dependent manner. Fig.7A shows western blot analysis carried out to characterize the MTA3 antibody and indicates the abundant amounts of MTA3 protein in MCF-7 cells. Since ER mediated activation of transcription involves recruitment of coactivators to the promoter area, we next examined the potential role of ER coactivators in the regulation of MTA3 expression. Using previously characterized MCF-7 cells stably expressing T7-PELP1 (also known as NMAR) (26), we showed that estrogen stimulation leads to enhanced recruitment of PELP1/NMAR to the MTA3 promoter chromatin (Fig. 7C), implying a role of coactivators in the regulation of MTA3 expression by ER. To determine the impact of MTA1 in the recruitment of coactivators to the MTA3 promoter chromatin, we next used MCF-7 cells expressing T7-MTA1 and examined the ability of estrogen to recruit AIB1, another ER-coactivator (27, 28), to the MTA3 promoter chromatin. As illustrated in Fig. 7D, upon estrogen stimulation, we found significant recruitment of endogenous AIB1 to the MTA3 promoter chromatin in MCF-7/vector cells. Deregulation of MTA1 completely abolished the noticed AIB1 interaction with the MTA3 promoter chromatin. To further understand the mechanistic participation of ER coactivators in the regulation of MTA3 expression, we next examined the ability of PELP1 and/or AIB1 to stimulate MTA3 transcription using promoter assays (Fig.7E). We observed a substantial induction of the MTA3 promoter activity by PELP1/MNAR or AIB1 in comparison with the vector- cotransfected cells. Coexpression of both PELP1/MNAR and AIB1 further augmented transcriptional activity of the MTA3 promoter as compared to expression of the individual

coactivators by themselves. Interestingly, overexpression of MTA1 completely inhibited the ability of PELP1/NMAR and/or AIB1 to stimulate MTA3 transcription.

In brief, these observations suggest that the MTA3 chromatin segment, containing the bona-fide ER recruitment site, represents a highly dynamic surface for ER as well as ER interacting coactivators and corepressors. The resulting transcriptional activity may be tightly regulated by the dynamic interplay of ER coregulators. Fujita et al. made an interesting observation that each of the MTA family members could be essential components of distinct subsets of the Mi-2/NuRD complexes that have unique functional properties (20). MTA1 repression of MTA3 expression could presumably be a mechanism by which the cell type-specific transcription is controlled. We hypothesize that the levels of MTA1 in the cell would determine which subset of the Mi-2/NuRD complex would be active to carry out its specialized function. Since MTA1 has been shown to be associated with more metastasis and invasiveness in tumors, regulation of MTA3 gene expression by MTA1, both under basal conditions as well as in the presence of estrogen, assumes importance. In this context, MTA1 overexpression, leading to the downregulation of MTA3 and E-cadherin expression, would give the tumors survival and metastatic advantage over other cells. In our model cell lines, overexpression of MTA1 resulted in non-responsiveness of these cells to estrogen in terms of induction of MTA3 gene by ER. It could be speculated that this could be one of the mechanisms by which tumors eventually stop responding to estrogen and consequently, anti-estrogen therapy. Furthermore, it is also possible that MTA1 may also repress gene expression of both ER and its coactivators in these situations. If it does so, then MTA1 could be targeted for therapeutic intervention in order to sensitize the tumor cells to anti-estrogen therapies, which could have tremendous clinical impact.

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FIGURE LEGEND

Fig. 1. Identification of the ER interaction site on the MTA3 regulatory elements. (A) Schematic representation of the MTA3 gene around the three possible ERE half-site -α recruitment sites. (B) Association of ER with the MTA3 chromatin. E2 signaling promotes interaction of ER with one of the possible ERE half-site in MTA3 chromatin. MCF-7 cells grown in a phenol red-free medium supplemented with 3% charcoal-dextran-stripped fetal bovine serum were treated with or without estrogen (10^{-9}M) for 60 min; chromatin lysates were immunoprecipitated with antibodies against ER, and samples were processed as described in "Experimental Procedure". The lower panel shows the PCR analysis of the input DNA of the MTA3 chromatin. The upper panel demonstrates the PCR analysis of the MTA3 promoter fragments for possible association with ER (n=3). (C) MCF7 cells were maintained in medium supplemented with 3% charcoal-dextran-stripped fetal bovine serum before treating either with estrogen (10^{-9} M), ICI-182780, both estrogen and ICI, cyclohexamide (10 μg/ ml) or cyclohexamide plus estrogen. The upper panel demonstrates the PCR analysis of the MTA3 promoter fragments for possible association with ER. The middle panel shows the PCR analysis of the input DNA of the MTA3 chromatin. The lower panel shows a Western blot analysis for ER under the same conditions (n=3).

Fig. 2. Estrogen-mediated induction of MTA3 promoter activity. (A) Induced luciferase activity with 735 base pair and 1078 base pair fragments of the MTA3 promoter (n=3) in MCF-7 cells. Cells were maintained in 3% DCC serum in phenol-red free medium for 48h before transfection of the luciferase constructs, luciferase activity was assayed at 48 hours post transfection. Values are normalized to β-Gal activity(n=3). (B) Induced luciferase activity with

735 base pair and 1078 base pair of the MTA3 promoter (n=3) in Hela cells. (C) and (D) Estrogen (10^{-9} M) and 4- hydroxyl tamoxifen (10^{-8}M) mediated regulation of MTA3 promoter activity in MCF7 cells and in Hela cells, respectively (n=3). All treatments with ligands were for 16 hours. (E) Schematic diagram of theMTA3 promoter (upper bar) was deleted and mutated. To find out which ERE half-site is responsible for the estrogen induction, a point mutation of the proximal ERE half site at -1241 is done (TGACC to TGCTC) (middle bar) and the second ERE half site is deleted at –1450 by PCR (bottom bar). (F) The 1078 base pair wild type MTA3 promoter as well as the deletion and point mutation constructs are transfected in MCF-7 cells and treated with estrogen as before. The point mutation at the proximal ERE half site abolishes the estrogen induction completely, where as the deletion of the middle ERE half site has little effect on estrogen stimulation

Fig. 3. MTA1 mediated interference of association of ER with the MTA3 chromatin. Cells were maintained in 3% charcoal-dextran-stripped fetal bovine serum before treating with estrogen (10^{-9} M) in the following experiments. (A) MTA1 inhibits ER recruitment on the MTA3 chromatin. The lower panel shows input DNA for the ChIP assay. MCF7 cells overexpressing pcDNA or T7-MTA1 were taken for the above experiment. (B) Short variant of MTA1 inhibits association of ER with the MTA3 chromatin. The lower panel shows the input DNA for the ChIP assay. ChIP assay was performed in the ZR-75R cells overexpressing vector alone, or T7-MTA1s using the anti ER antibody. (C) RT-PCR analysis of the MTA3 mRNA level in MCF7 cells stably expressing either control vector or T7-MTA1. (D) MTA3 transcription activity in the presence of either control vector or T7- MTA1 with or without estrogen (10^{-9} M, 16 h).

Fig. 4. Expression of MTA3 and its downstream effectors in MTA1 overexpressing cells by Confocal microscopy. Confocal analysis of E-cadherin (upper panel), MTA3 and Snail in MCF-7 cells expressing pcDNA (middle panel) and MTA1 (lower panel). In the upper panel, E-cadherin was stained red and in the lower panel, MTA3 was stained green and Snail was red. The nuclei were visualized using Topro3(blue). The cells were all cultured in 10% serum conatining medium.

Fig. 5. Direct association of MTA1 with the MTA3 chromatin and consequence of MTA1 and ER silencing on molecules downstream of MTA3. (A) ChIP analysis of direct association of MTA1 with the MTA3 chromatin in the presence or absence of estrogen. ChIP analysis was performed in MCF-7 cells. (B) ChIP analysis of recruitment of HDAC1 and HDAC2on the MTA3 chromatin in MCF-7 cells in the presence or absence of estrogen. (C) Western analysis of molecules downstream of MTA3 as a result of knocking down MTA1 by siRNA in MCF7 cells. The same blot was stripped and analyzed for expression of Snail, E-cadherin, PR-A and PR-B as well as vinculin as a loading control. (D) RT-PCR analysis of the MTA3 mRNA level in MCF-7 cells as a result of MTA1 silencing with or without estrogen.

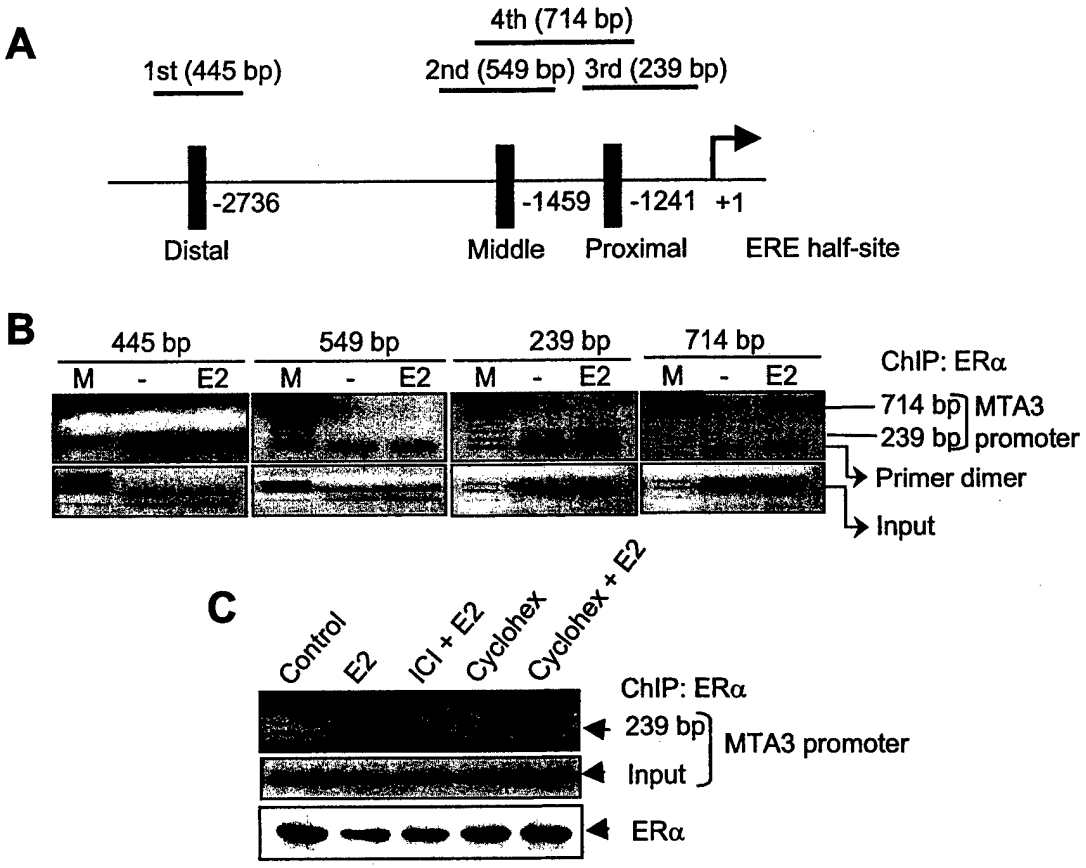
Fig. 6. Effect of MTA1 knockdown on MTA3 gene regulation (A) ChIP analysis of the MTA3 chromatin for ER recruitment in MCF7 cells with or without MTA1 silencing in the presence or absence of estrogen (10^{-9}M). (B) ChIP analysis of the MTA3 chromatin for HDAC1 and HDAC2 recruitment in the presence or absence of estrogen in MCF-7 cells having normal or silenced MTA1 expression. (C) Effect of MTA1 silencing on MTA3 luciferase activity in MCF7

cells in the presence or absence of estrogen (10^{-9} M). (D) Immunoblotting of samples having normal ER-α expression and silenced ER expression, which is reprobed for PR (PR-A and PR-B) and vinculin as a loading control. (E) ER was knocked down in MCF7 cells, and ChIP analysis was performed for T7-MTA1 recruitment on the MTA3 chromatin in the presence or absence of estrogen.

Fig. 7. Coregulator recruitment and its effect on MTA3 promoter activity (A) Detection of MTA3 protein in MCF-7 cell line. (B) Increased expression of MTA3 protein in MCF-7 cells in response to different concentrations of estrogen after 24 hours of treatment. MTA3 protein levels was quantified using ImageQuant and normalised to vinculin levels in the cells. (C) Association of PELP1 with MTA3 promoter in a time dependent and estrogen-sensitive manner in MCF-7 cells stably expressing T7-PELP1. For immunoprecipitation, anti-T7 antibody was used, which is targeted against the T7-PELP1. (D) MTA1 inhibits AIB1's association with the MTA3 chromatin. MCF-7 cells either overexpressing the vector alone or T7-MTA1 were analyzed by ChIP assay for possible recruitment of AIB1 with or without estrogen signaling. Cross-linked cells were lysed and taken for immunoprecipitation with anti-AIB1 antibody. (E) MTA3 luciferase activity was measured in the presence or absence of different coactivators, such as AIB1 and PELP1, and the corepressor MTA1 with or without estrogen treatment.

Table I. Primer and siRNA sequences used in the study

MTA3 promoter cloning	
MTA3-pro1988F	tgtcagagagctcttggtgggatctctggta
MTA3-pro3066R	gagcctcgaggctctagggccaggaa
MTA3-pro2331F	ttatttgagctctttgcctcagctatgca
MTA3-pro3066R	gagcctcgaggctctagggccaggaa
MTA3 promoter del/mutation	
MTA3pro2073F	tgacacagagctcagaatttgacacac
MTA3pr-mut-F2	acgaggaaacagagcatagagtca
MTA3pr-mut-R2	tgactctatgctctgtttcctcgt
MTA3 RT-PCR F	accctcgtgttagaagtcacgtgt
MTA3 RT-PCR R	gcagcataattaatagcaacaaacgg
MTA3 cDNA cloning	
MTA3-start	gcgggtacccatggcggccaacatgtaccgggt
MTA3-stop	caacatctcgagttaagaatttaaaagcatct
MTA3 ChIP PRIMERS	
MTA3ChIPF1	ggaatagagagaagggacctaacgc
MTA3ChIPR1	tgagcctcaagaggggttacaa
MTA3ChIPF2	cataagcaatttctcctccttcgaa
MTAChIPR2	tcacgtccccattttatagacgag
MTAChIPR3	acacagctgtgtgctgtcgact
MTAChIPF4	tggtttctaggtggcttttg
MTAChIPR4	tggtggcttgtttggaatgt
MTA1 siRNA	
Mta1siRNA-1	aaccctgtcagtctgctataa
Mta1siRNA-2	aagaccctgctggcagataaa
Mta1siRNA-3	aagattttctcccgtggaagt
Mta1siRNA-4	aagaagcgcggctaacttatt
Control siRNA	Nonspecific pooled duplex control Catalog number: SD-001206-13-80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1st (445 bp)
2nd (549 bp)
3rd (239 bp)
-2736
-1459
-1241
+1
Distal
Middle
Proximal
ERE half-site
B
445 bp
549 bp
239 bp
714 bp
M - E2
ChIP: ERα
714 bp
239 bp
MTA3 promoter
Primer dimer
Input
C
Control
E2
ICI + E2
Cyclohex
Cyclohex + E2
ChIP: ERα
239 bp
Input
MTA3 promoter
ERα

Fig. 1

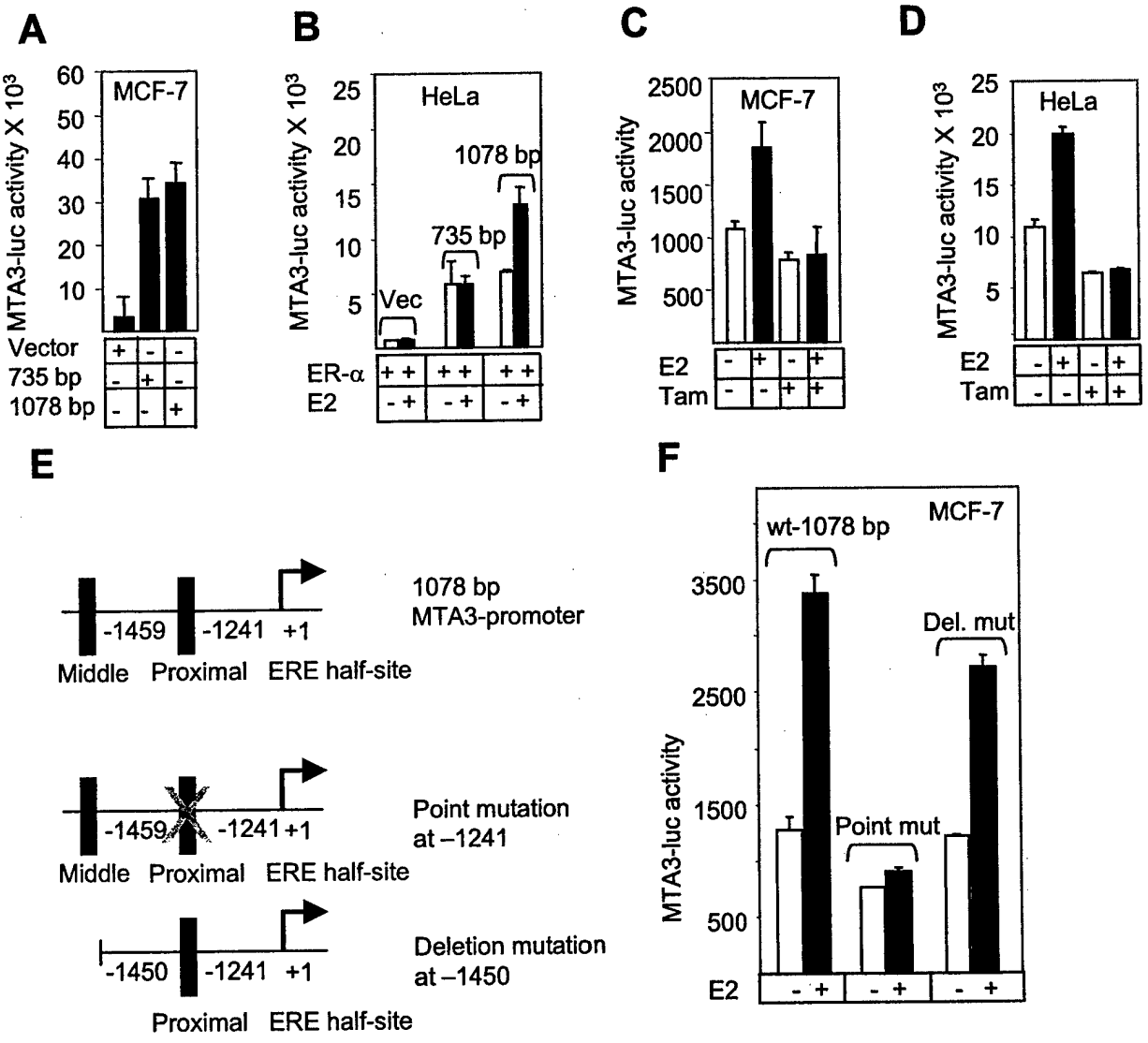
A
MTA3-luc activity X 10³
MCF-7
Vector
735 bp
1078 bp
B
MTA3-luc activity X 10³
HeLa
1078 bp
735 bp
Vec
ER-α
E2
C
MTA3-luc activity
MCF-7
E2
Tam
D
MTA3-luc activity X 10³
HeLa
E2
Tam
E
1078 bp
MTA3-promoter
-1459
-1241
+1
Middle
Proximal
ERE half-site
Point mutation
at –1241
Deletion mutation
at –1450
-1450
F
wt-1078 bp
MCF-7
Del. mut
Point mut
MTA3-luc activity
E2

Fig 2

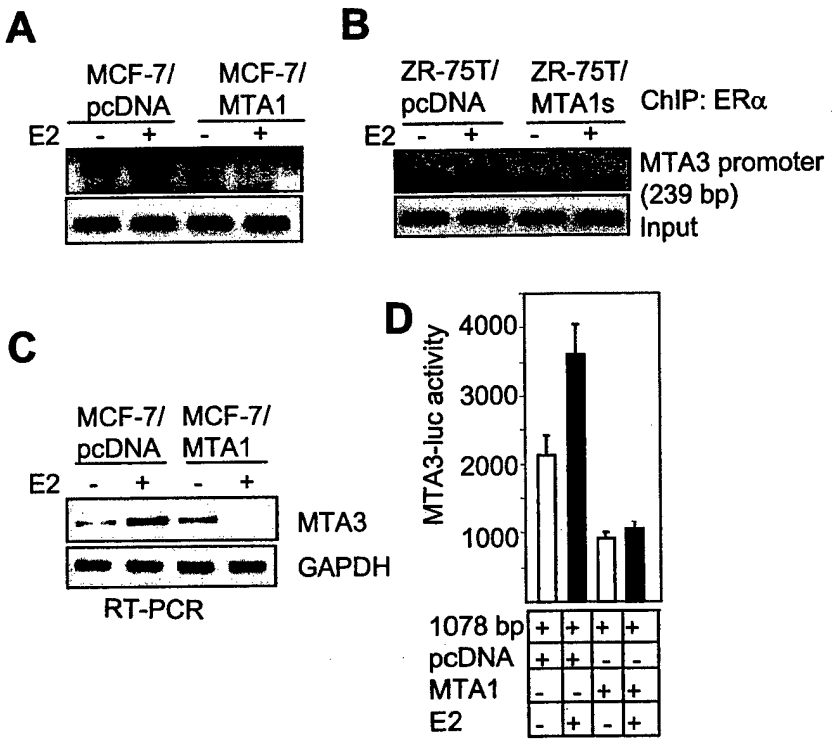
A
MCF-7/ pcDNA
MCF-7/ MTA1
E2 - + - +
B
ZR-75T/ pcDNA
ZR-75T/ MTA1s
ChIP: ERα
E2 - + - +
MTA3 promoter (239 bp)
Input
C
MCF-7/ pcDNA
MCF-7/ MTA1
E2 - + - +
MTA3
GAPDH
RT-PCR
D
MTA3-luc activity
4000
3000
2000
1000
1078 bp + + + +
pcDNA + + - -
MTA1 - - + +
E2 - + - +

Fig 3

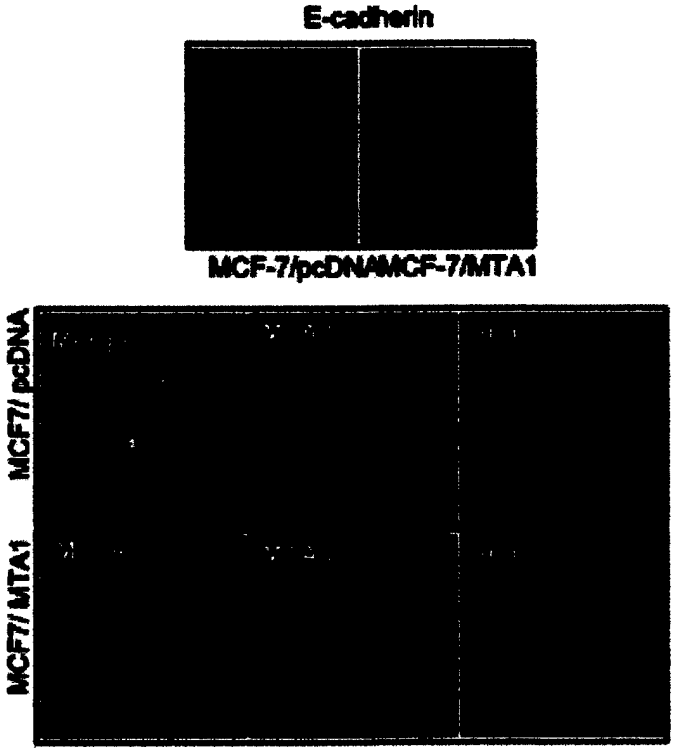

Fig 4

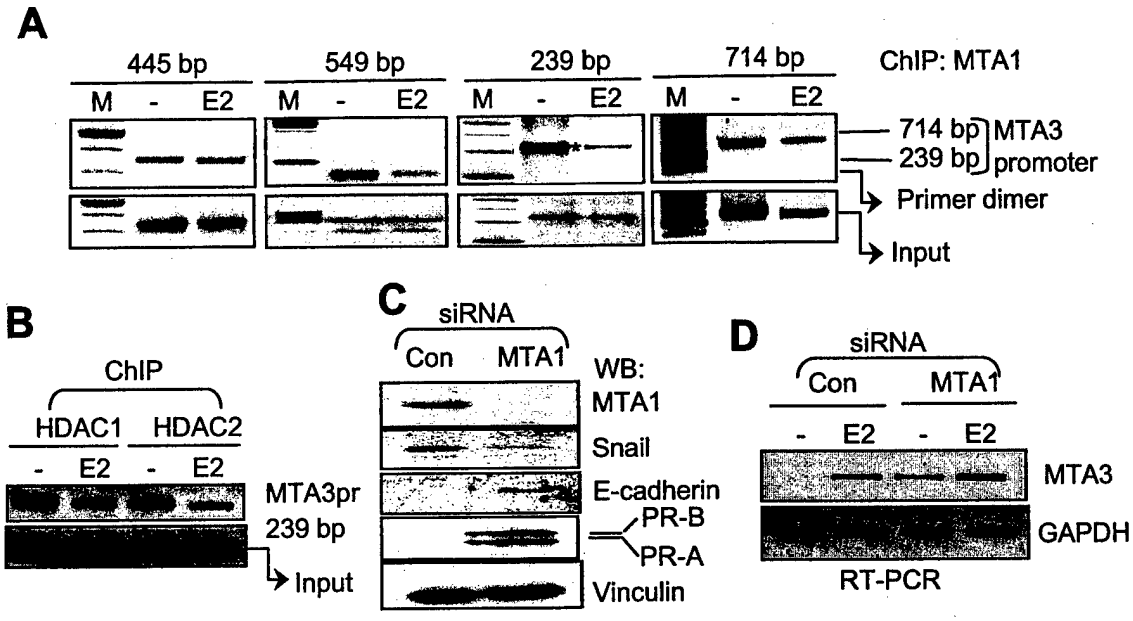
A
445 bp
549 bp
239 bp
714 bp
ChIP: MTA1
M - E2
714 bp
239 bp
MTA3 promoter
Primer dimer
Input
B
ChIP
HDAC1 HDAC2
- E2 - E2
MTA3pr 239 bp
Input
C
siRNA
Con MTA1
WB:
MTA1
Snail
E-cadherin
PR-B
PR-A
Vinculin
D
siRNA
Con MTA1
- E2 - E2
MTA3
GAPDH
RT-PCR

Fig 5

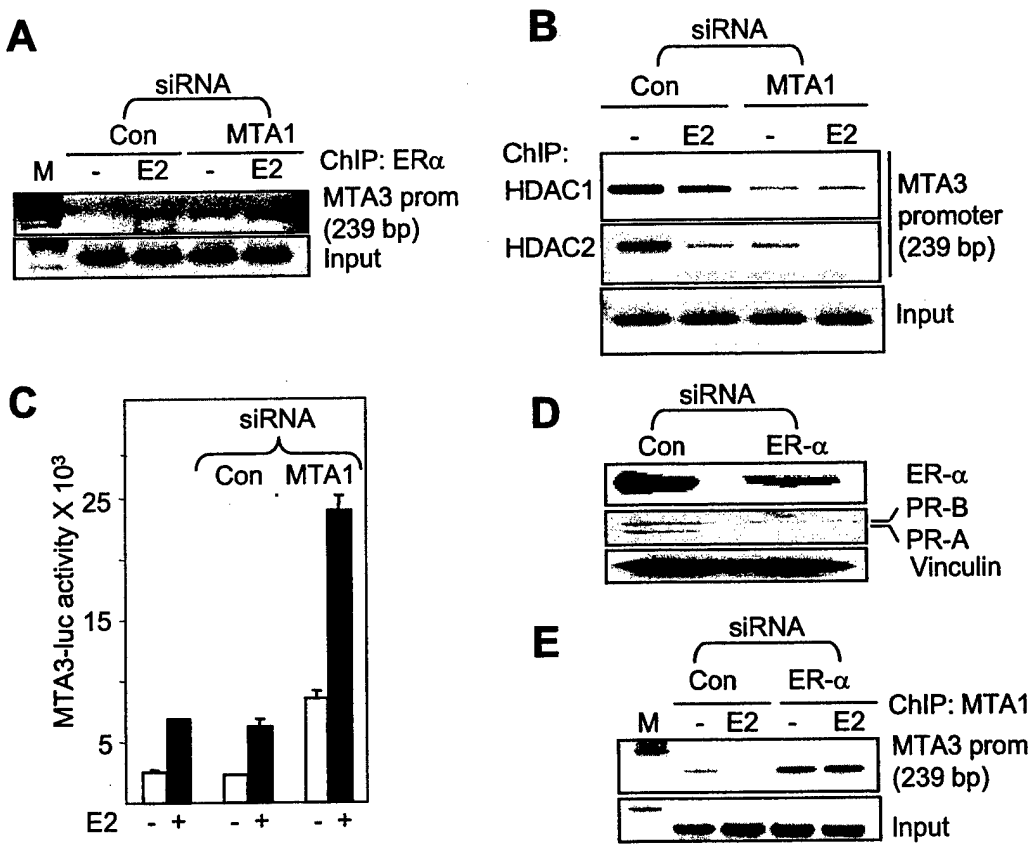
A
siRNA
Con
MTA1
M
-
E2
-
E2
ChIP: ERα
MTA3 prom
(239 bp)
Input
B
siRNA
Con
MTA1
-
E2
-
E2
ChIP:
HDAC1
HDAC2
MTA3
promoter
(239 bp)
Input
C
siRNA
Con
MTA1
MTA3-luc activity X 10³
25
15
5
E2
-
+
-
+
-
+
D
siRNA
Con
ER-α
ER-α
PR-B
PR-A
Vinculin
E
siRNA
Con
ER-α
ChIP: MTA1
M
-
E2
-
E2
MTA3 prom
(239 bp)
Input

Fig 6

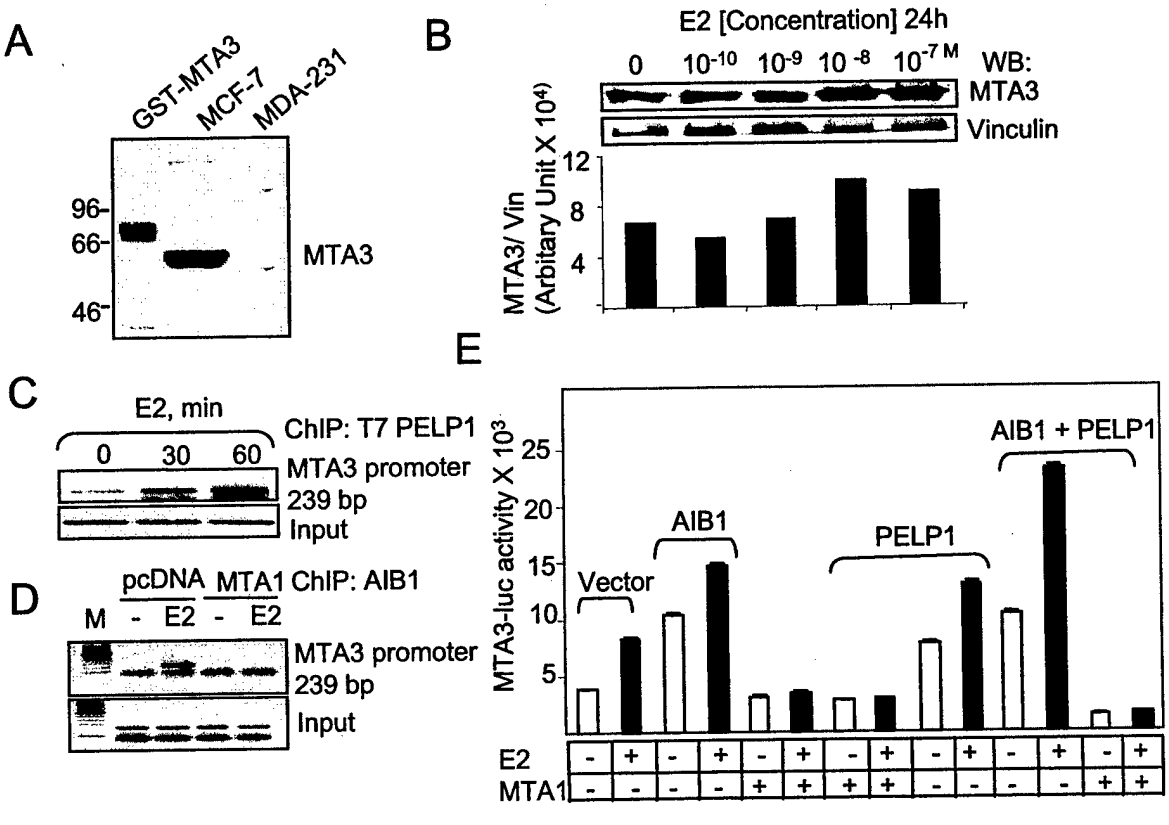
A
GST-MTA3
MCF-7
MDA-231
96-
66-
46-
MTA3
B
E2 [Concentration] 24h
0
10-10
10-9
10 -8
10-7 M
WB:
MTA3
Vinculin
MTA3/ Vin
(Arbitary Unit X 10⁴)
12
8
4
C
E2, min
0
30
60
ChIP: T7 PELP1
MTA3 promoter
239 bp
Input
D
pcDNA
MTA1
ChIP: AIB1
M
-
E2
-
E2
MTA3 promoter
239 bp
Input
E
MTA3-luc activity X 10³
25
20
15
10
5
Vector
AIB1
PELP1
AIB1 + PELP1
E2
MTA1

Fig 7